Phylogeny of the Genus *Pomatocalpa* Breda (Orchidaceae)

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Abstract

The phylogeny of the orchid genus *Pomatocalpa* has been analyzed using morphological and molecular (*matK* and ITS) data. First, 9 representative species of *Pomatocalpa*, viz. *P. armigerum* (King & Pantl.) T.Tang & F.Wang, *P. bambusarum* (King & Pantl.) Garay, *P. bhutanicum* N.P.Balakr., *P. bicolor* (Lindl.) J.J.Sm., *P. spicatum* Breda, *P. kunstleri* (Hook.f.) J.J.Sm., *P. diffusum* Breda, *P. macphersonii* (F.Muell.) T.E. Hunt and *P. tonkinense* (Gagnep.) Seidenf. and 11 neighboring genera (according to morphological features), viz. *Acampe*, *Cleisostoma*, *Micropera*, *Haraella*, *Pelathanteria*, *Robiquetia*, *Sarcoglyphis*, *Smitinandia*, *Staurochilus*, *Trichoglottis* and *Ventricularia,* were included to test the monophyly and phylogenetic position of the genus *Pomatocalpa*. This analysis was based on morphological data only, and the genera *Seidenfadenia*, *Vanda* and *Ascocentrum* were chosen as outgroups. Second, the interspecific phylogenetic relationships within *Pomatocalpa* were analyzed on the basis of *matK* and ITS sequences and morphological characters. The molecular analyses included seven species of *Pomatocalpa*, viz. *P. undulatum* (Lindl.) J.J.Sm., *P. bicolor, P. diffusum, P. kunstleri*, *P. macphersonii*, *P. maculosum* (Lindl.) J.J.Sm. and *P. spicatum*, and used *Acampe ochracea* (Lindl.) Hochr., *Ventricularia tenuicaulis* (Hook.f.) Garay and *Smitinandia micrantha* (Lindl.) Holtt. as outgroups. The morphological analysis included all 13 accepted species of *Pomatocalpa* by us and used

Acampe, *Ventricularia*, and *Smitinandia* as outgroups. Based on the results of an unpublished precursory study, the monotypic genus *Haraella* was included in the ingroup in both the morphological and molecular analyses. In addition to the separate analyses, a total evidence analysis was made, combining information from *matK*, ITS, and morphological data. *Pomatocalpa* turned out to be monophyletic, but only after exclusion of *P. armigerum* (= *Cleisostoma armigerum* King & Pantl.), *P. bambusarum* (= *Cleisostoma bambusarum* (King & Pantl.) King & Pantl.) and *P. bhutanica* (= *Cleisostoma bhutanicum* (N.P.Balakr.) S. Watthana, comb. nov.). According to *matK*, morphology, and total evidence, *Pomatocalpa* is monophyletic without *Haraella*. According to ITS data, on the other hand, *Haraella* is nested in *Pomatocalpa*. The incongruence of *matK* and ITS sequences may be because *Haraella retrocalla* is of ancient hybrid origin. When delimited according to this paper, the genus *Pomatocalpa* is probably monophyletic and is characterized by a single unique synapomorphy, i.e., the presence of a narrow longitudinal groove at the upper end of the front wall of the spur. The interspecific relationships in *Pomatocalpa* were poorly resolved.

Introduction

The orchid genus *Pomatocalpa* Breda belongs to subtribe Aeridinae, subfam. Epidendroideae, tribe Vandeae (Dressler, 1993). It contains 13 species distributed from Sri Lanka to Fiji, south to Northern Australia and north to Hainan and Taiwan (Watthana, in prep.).

A comprehensive hypothesis about the phylogeny of the genus *Pomatocalpa* is not yet available. A recent global phylogenetic analysis of subtribe Aeridinae based on DNA (*matK* and ITS sequences) contributes some information (Topik et al., 2005). In that analysis, *Pomatocalpa* is represented by *P. diffusum* and *P. kunstleri*. On this limited basis, *Pomatocalpa* appears to be monophyletic with the monotypic genus *Haraella* as a sister-group.

As a precursory work of the present study, the molecular phylogeny of the Aeridinae has been re-analyzed with additionally seven species of *Pomatocalpa* included, which were available for DNA extraction only, viz *P. undulatum* s.l. (including *P. acuminatum* (Rolfe) Schltr.), *P. bicolor*, *P. diffusum*, *P. kunstleri*, *P. macphersonii*, *P. maculosum* and *P. spicatum* (Topik and Yukawa, unpublished). However, they seem to represent almost all the variations observed in the genus. In this analysis, *Pomatocalpa* is still

monophyletic according to *matK* data. In the re-analysis based on ITS data, on the other hand, *Haraella* is nested in *Pomatocalpa*.

The objectives of the present study are: 1) to test the monophyly of *Pomatocalpa* more thoroughly and to identify its phylogenetic position among closely related genera according to morphological features; 2) to resolve the interspecific phylogenetic relationships in *Pomatocalpa* based on molecular and morphological data and on total evidence.

Materials & Methods

I. Delimitation and phylogenetic position of Pomatocalpa (morphological data)

A. Sampling

Acampe, *Cleisostoma*, *Haraella*, *Micropera*, *Pelathanteria*, *Robiquetia*, *Sarcoglyphis*, *Smitinandia*, *Staurochilus*, *Trichoglottis* and *Ventricularia* were identified as the genera being morphologically most similar to *Pomatocalpa*. Thus, all of them except *Robiquetia* are placed in the same main group in Seidenfaden's (1988) comprehensive account of Thai monopodial orchid genera; additionally, certain species of *Robiquetia* and *Cleisostoma* have previously been referred to *Pomatocalpa*. Consequently, these genera were included in a phylogenetic analysis together with nine representative species of *Pomatocalpa*, viz. *P. armigerum*, *P. bambusarum*, *P. bhutanicum*, *P. bicolor*, *P. spicatum*, *P. kunstleri*, *P. diffusum*, *P. maphersonii* and *P. tonkinense*. Furthermore, *Trichoglottis lasiocarpa* was treated separately from the rest of *Trichoglottis* in the analysis, because this species was only recently transferred to the latter genus from *Pomatocalpa* (Ormerod, 1997). Unfortunately, no specimen of *Robiquetia vaupelii* (syn. *Pomatocalpa vaupelii* Ormerod & J. J. Wood) was available for this study. The genera *Seidenfadenia*, *Vanda* and *Ascocentrum* were chosen as outgroups. According to the molecular phylogenetic study of Topik et al. (2005), these three genera belong to the clade that is sister to the clade accommodating the genera of the ingroup of the present study.

The decision to include genera as terminal taxa in the ingroup was made because this practice was expected to increase the influence of ancestral character states by reducing the noise from later-evolved species-specific features (Wiens, 2000). Naturally, the best solution would have been to include all individual species of all the genera concerned. However, this

would have become a major project, far beyond the scope of the present study. The only realistic alternative would be to include one or few representative species from each genus. Since none of the genera concerned have ever been thoroughly analyzed phylogenetically, a more or less random selection of "representative" species might well influence the analysis through the differences in later-evolved species-specific features. Naturally, we realize that including the genera as terminal taxa in the ingroup probably reduces the final resolution. Still we consider this practice to be the better option.

B. *Characters*

Morphological characters are usually regarded as qualitative or quantitative. However, so-called qualitative characters often have a quantitatively phenomenological base (Stevens, 1991) and can be expressed quantitatively by the systematist coding them (Baum, 1988). According to Stevens (1991), character states used in phylogenetic analysis should be discrete and based on carefully analyzed discontinuities in the variation. However, it seems that neither characters with a continuous variation pattern, nor characters with overlapping attribute values of the taxa, should be excluded a priori from phylogenetic analyses. Thiele (1993) and Ryding (1998) found such polymorphic characters to track phylogeny almost as accurately as characters with a discontinuous variation pattern. The results of Wiens (1995) suggest that polymorphic characters may contribute significant phylogenetic information, but also that they are less reliable. On this background, it was decided to include polymorphic characters in the present study.

For all genera included in the analysis, the character states were scored from: (1) the keys and descriptions in Schuiteman & de Vogel (2000), Seidenfaden (1988) and Seidenfaden & Wood (1992); (2) herbarium specimens and spirit samples deposited at C; (3) live specimens cultivated in the Botanical Garden, University of Copenhagen.

Some of the measurements are defined in Figure. 1. For this part of the study, 18 characters were scored (Table 1) and the matrix is shown in Table 2. The inflorescence type was excluded due to occasional variation within individual specimens of certain species (e.g., *Pomatocalpa bicolor*, *P. diffusum*, *P. kunstleri* and *P. spicatum*). It should also be mentioned that several floral characters were excluded because of too much variations seen in many of the terminal taxa.

The interpretation of the variation encountered in some of the

accepted characters should be briefly explained.

Habit (character 1). Plants with moderately elongate stems carrying many condensed leaves (e.g., *Vanda*) were coded as "stem short". Certain species of *Pomatocalpa*, e.g. *P. diffusum*, are polymorphic. However, *P. kunstleri*, *P. macphersonii* and *P. spicatum* consistently have a short stem, while *P. bicolor* consistently has a rambling habit with an elongate stem.

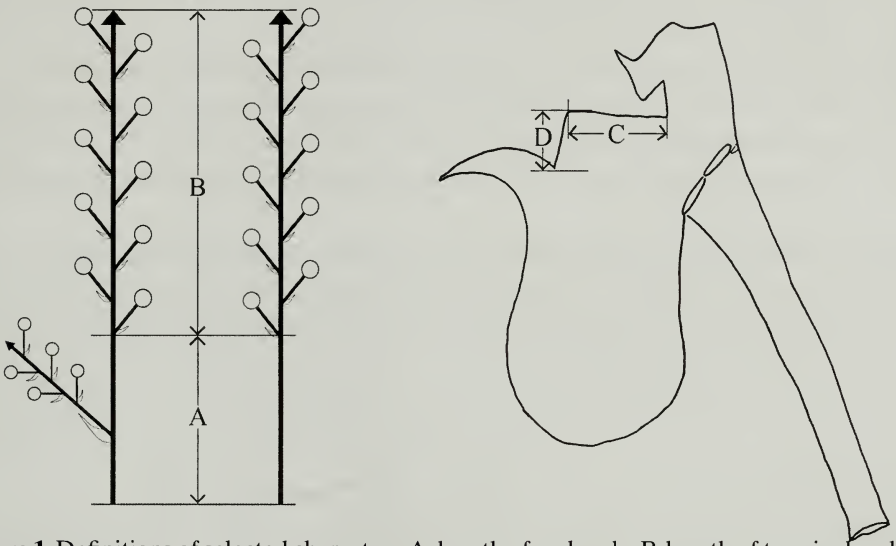

Figure 1. Definitions of selected characters. A, length of peduncle; B, length of terminal rachis shoot; C, hind edge of labellum side lobe; D, front edge of labellum side lobe.

Table 1. List of morphological characters and states used in the phylogenetic analysis of intergeneric relationships.

1. Stem elongate (0) or short (1).
2. Labellum subglabous to minutely papillose (0) or pubescent to distinctly hairy (1).
3. Mid-lobe without (0) or with ornaments (1).
4. Mid-lobe simple (0) or lobed (1).
5. Spur absent (0) or present (1).
6. Spur without (0) or with (1) a median septum.
7. Front wall of spur with (0) or without (1) a narrow longitudinal groove at its upper end.
8. Front wall of spur without (0) or with (1) a big fleshy callus at its upper end.
9. Back wall of spur without (0) or with (1) an ornament (callus or tongue).

10. Back wall ornament situated near the spur entrance (0) or down in the spur (1).
11. Back wall ornament a fleshy callus (0) or a membranous tongue (1).
12. Back wall tongue glabrous (0) or hairy (1).
13. Stipe broadly rectangular to subspathulate (0), (linear-)oblong (1) or narrowly linear (2).
14. Viscidium large and broader than long (0); large and ovate to oblong (1) or small and subglobose (2).
15. Pollinia two (0) or four (1).
16. Rostellum shorter (0) or longer (1) than the diameter of column.
17. Column not dorsally prolonged and incurved (0) or column dorsally prolonged and incurved (1).
18. Column without (0) or with (1) two horizontally protruding appendages.

Table 2. Character by taxon matrix of the analysis of intergeneric relationships. Characters as in **Table 1**. ?=unknown, -=inapplicable. Polymorphic taxa are coded with the following symbols; these are equate macros in PAUP: b = (0&1).

	5	10	15	
Seidenfadenia	00001	0000-	--100	000
Vanda	00101	00b0-	--120	000
Ascocentrum	00001	00b0-	--1b0	000
Acampe	11101	0000-	--101	000
Smitinandia	10001	0010-	--021	000
Venticularia	11101	0100-	--001	000
Trichoglottis	11bb1	0001b	1b101	000
T. lasiocarpum	01111	00011	11101	000
Staurochilus	11b1b	0001b	11101	000
Cleisostoma	bbbb1	b001b	0-bb1	b00
Pelathanteria	1bbb1	1001b	0-021	000
Micropera	1b1b1	101bb	b-111	100
Robiquetia	b0b01	000bb	b-1b0	bbb
Sarcoglyphis	100b1	100bb	b-111	000
Haraella	01000	-----	--100	000
P. bhutanicum	1?101	00010	0-101	000
P. armigerum	11101	00011	0-101	000
P. bambusarum	00101	00111	10111	111
P. bicolor	10001	01011	10101	000
P. spicatum	00001	01011	10101	000
P. kunstleri	00001	01011	10101	000
P. diffusum	b0001	01011	10101	010
P. maphersonii	00001	01011	10101	010
P. tonkinense	b0001	01011	10101	010

Labellum surface (character 2). Due to the homogeneity of the epidermis (and due to intermediary states), the condition of a densely and minutely papillose surface (e.g. *P. diffusum* and some specimens of *P. macphersonii*) was considered conceptually similar to a subglabrous surface (e.g. *P. spicatum* and *P. bicolor*). Consequently, these conditions were treated as one character state and opposed to the condition of a properly hairy surface with heterogenous epidermis (e.g. in *Acampe*, *Haraella*, *Staurochilus* and *Trichoglottis*).

Ornaments on the labellum mid-lobe (character 3). In this context, the term "ornaments" covers a range of morphologically diverse structures, such as keels, warts, or markedly thickened margins. It is uncertain to which extent these structures are homologous.

Front wall groove (character 7). Several species and genera have two small calli at the boundary between the base of the labellum mid-lobe and the upper end of the front wall of the spur. However, only in some (but not all) species traditionally assigned to *Pomatocalpa*, these calli are adjoining, elongate and extend down into the spur, thus producing a narrow longitudinal groove of varying length between them.

Front wall callus (character 8). The presence of two tiny calli at the boundary between the base of the labellum mid-lobe and the upper end of the front wall of the spur is common among the group of genera examined. However, this feature is markedly different from the presence of one big fleshy callus – a character state only observed in *Pomatocalpa bambusarum*, *Micropera* and *Smitinandia*. In the latter genus, it varies from globular (*S. micrantha*) to somewhat complanate (*S. helferi* (Hook.f.) Garay).

C. *Phylogenetic analyses*

The phylogenetic analysis based on maximum parsimony (MP) was performed using PAUP* version 4.0b10 for Microsoft Windows (Swofford, 2001). All characters were equally weighted, unordered (Fitch, 1971). The data set was analysed by the heuristic search method with bisection-reconnection (TBR) branch swapping and the MULTREES option on, saving all most parsimonious trees (MPTs). Evaluation of internal support of clades was conducted by the bootstrap analysis (Felsenstein, 1985) with 10,000 replicates with faststep searching.

Interspecific Relationships in *Pomatocalpa*

A. Molecular data

A.1. *Sampling*

For this part of the study, matK and ITS sequences could be obtained from seven species of Pomatocalpa, viz. *P. undulatum, P. bicolor, P. diffusum, P. kunstleri, P. macphersonii, P. maculosum* (Lindl.) J.J.Sm. and *P. spicatum.* Due to the results of a preliminary analysis encompassing many more taxa of subtribe Aeridinae (cf. the Introduction), *Haraella retrocalla* (Hayata) Kudo (the only species of Haraella) was included in the ingroup, while *Acampe ochracea, Smitinandia micrantha* and *Ventricularia tenuicaulis* were used as outgroups.

Material of *Pomatocalpa* was partly collected in the field in Thailand by the first author and partly obtained from living collection in the Tsukuba Botanic Garden, Japan and from Queen Sirikit Botanic Garden, Chiang Mai, Thailand. A list of voucher specimens is given in **Table 3**.

Table 3. List of species analysed in the molecular analyses.

Species	Voucher	Source
Pomatocalpa diffusum Breda	Watthana 1767 (QBG)	Thailand
P. bicolor (Lindl.) J.J. Sm.	Watthana 1821 (QBG)	Malaysia
P. maculosum (Lindl.) J.J.Sm.	Watthana 1768 (QBG)	Thailand
P. spicatum Breda	Watthana 1771 (QBG)	Thailand
P. kunstleri (Hook.f.) J.J.Sm.	TBG 145833 (TBG)	Borneo
P. undulatum (Lindl.) J.J.Sm.	Yukawa s.n. (TBG)	Taiwan
P. macphersonii (F.Muell.) Hunt	TBG 130189 (TBG)	Unknown
Acampe ochracea (Lindl.) Hochr.	TBG 130163 (TBG)	unknown
Haraella retrocalla (Hayata) Kudo	TBG 133078 (TBG)	Taiwan
Smitinandia micrantha (Lindl.) Holttum	TBG 118427 (TBG)	Vietnam
Ventricularia tenuicaulis (Hook.f.) Garay	TBG 145846 (TBG)	Thailand

A.2. *Nucleotides preparation*

The total DNA was extracted from fresh material or silica-gel dried plant tissues following the instruction of QIAGEN DNeasy Mini Plant Kit. For *matK*

sequences, the amplification was performed using a primer pair, OMAT1F and trnK-2R (Topik et al. 2005). The 20-µl amplification reaction included 2 µl 10 x of Ex-Taq buffer (Takara Bio Inc.), 1.6 µl 2.5 mM of dNTPs mix, 0.5 µl each primer (10 pmol), 0.1 micro liter 5 units/µl of Ex-Taq DNA-polymerase (Takara Bio Inc.), 2 µl of template DNAs and 13.3 µl of MilliQ water. The polymerase chain reaction (PCR) profile consisted of an initial 5 min premelt at 94°C and 30 cycles of 30 s at 94°C, 30 s at 53°C, and 3 min at 72°C, followed by a final 7 min extension at 72°C.

Amplification of the ITS region was carried out using a primer pair, 17SE and 26SE (Sun et al., 1994). Total volume of PCR was 30 µl that included 15 µl GC buffer I (Takara Bio Inc.), 4.8 µl 2.5 mM of dNTPs mix, 0.5 µl of each primer (10 pmol), 0.21 µl 5 units/µl of LA Taq DNA-polymerase (Takara Bio Inc.), 2.4 µl of template DNAs and 6.59 µl of MilliQ water. The PCR profile consisted of an initial 2 min premelt at 94°C and 30 cycles of 50 s at 94°C, 1 min at 60°C, and 30 s at 72°C, followed by a final 7 min extension at 72°C. To confirm the number of amplified copies for ITS regions, we performed the single-strand conformation polymorphism (SSCP) analysis based on the method developed by Orita et al. (1989).

The PCR products were cleaned by using Montaqe PCR Centrifugal Filter Devices (Millipore Co.) and were used for auto-cycle sequencing reaction. The 10-µl auto-cycle sequencing reaction included 3 µl of Master Mix (Beckman Coulter), 1 µl primer (1.6 pmol), and 6 µl of PCR product. The reaction was incubated with 50 cycles of 20-s at 96°C, 20-s at 50°C and 4-min at 60°C.

Auto-cycle sequencing products were cleaned by adding STOP solution (2 µl 3M of NaOAc, 2 µl 100 mM of EDTA, and 1 µl 20 ng/µl of Glycogen) and 60 µl of 100 % ethanol; subsequently, they were centrifuged at 14000 rpm for 15-min at 4°C. The alcohol/salt mix was discarded, and the pellet was subjected to two washes with 200 µl 70% ethanol, each followed by centrifugation at 14000 rpm for 2-min at 4°C. Cleaned auto-cycle products were allowed to dry in the vacuum dry for 15-min. Both forward and reverse sequences were analyzed with CEQ8000 automated sequencer (Beckman Coulter), and electropherograms were edited and assembled with Genetyx-ATGC version 4.1 (Genetyx Corporation).

A.3. *Phylogenetic analyses*

DNA sequences obtained from *matK* and ITS were aligned with ClustalX and were then adjusted manually. Phylogenetic analysis and the evaluation of internal support of clades were performed by the same method as described for the morphological intergeneric analysis (see above).

B. Morphology

B.1. *Sampling*

Nearly all recently accepted species of *Pomatocalpa* by us were included in the ingroup. However, *P. armigerum*, *P. bambusarum* and *P. bhutanicum* were excluded because of the results from the analysis of the systematic position and delimitation of *Pomatocalpa* (see below). Due to considerations discussed above, also *Haraella retrocalla* was included in the ingroup, while the genera *Acampe*, *Smitinandia* and *Ventricularia* were chosen as outgroups. We preferred to use entire genera as outgroups (instead of using representative species of the same genera). This practice was expected to increase the influence from ancestral character states in the outgroup by reducing the noise from late-evolved species-specific features (Wiens, 2000).

Most of the data were obtained by examination of spirit and herbarium material from AAU, AMES, BM, C, K, L, and P. Additionally, the following publications were consulted: Seidenfaden (1988, 1992), Comber (1990), Seidenfaden and Wood (1992).

B.2. *Characters*

The choice of characters reflects the same general considerations as discussed under "Delimitation and phylogenetic position of *Pomatocalpa*" above. For this part of the study, 32 characters were scored (**Table 4**) and the matrix is shown in **Table 5**. Some of the characters are defined in **Figure 1**. Several leaf characters (morphology of the sheath, dimensions and shape of the blade, etc.) as well as the outline of the back wall tongue of the spur were excluded, mainly due to high levels of intraspecific variation. The interpretation of the variation encountered in some of the characters should be briefly explained.

Stem length (character 1). Kerr (1985) made a distinction between *Pomatocalpa* species with a compact fan-shaped habit and those with an elongate rambling habit. However, intermediary states are found in *P. diffusum*, *P. fuscum* (Lindl.) J.J.Sm. and *P. marsupiale* (Kraenzl.) J.J.Sm. Therefore, the length of stem was applied instead.

Length of peduncle in relation to rachis (character 3). This character is controversial in species with branched inflorescences. Furthermore, *P. spicatum* may produce both unbranched and branched inflorescences.

However, if the peduncle is defined to end at the point where the terminal rachis shoot starts, a ratio can be obtained that is comparable to the peduncle: rachis ratio in an unbranched inflorescence (**Figure 1**).

Hairiness of peduncle (character 4). According to Seidenfaden (1988), *P. maculosum* (subsyn. *P. linearifolium* Seidenf.) has a finely pubescent rachis. Judging from the first author's observation, it varies from minutely papillose to sparsely and finely pubescent on the rachis, but it is not pubescent on the peduncle. Only *P. kunstleri* has a finely pubescent peduncle.

Table 4. List of morphological characters and states used in the phylogenetic analysis of interspecific relationships in *Pomatocalpa*.

1. Stem up to 30 cm long (0) or more than 30 cm long (1).
2. Inflorescence erect (0) or horizontal to pendent (1).
3. Peduncle shorter than or as long as the terminal rachis shoot (0) or peduncle much longer than the terminal rachis shoot (1) – cf. **Figure 1**.
4. Peduncle glabrous to minutely papillose (0) or distinctly pubescent (1).
5. Inflorescence with less than 5 flowers (0), 5-30 flowers (1) or more than 30 flowers (2).
6. Apex of floral bract obtuse to acute (0) or acuminate to caudate (1).
7. Pedicel less than 5.0 mm long (0) 5.0-10.0 mm long (1) or more than 10.0 mm long (2).
8. Dorsal sepal up to 6.0 mm long (0) or more than 6.0 mm long (1).
9. Dorsal sepal without markings (0), (sub)bordered (1), spotted or patched (2), with two longitudinal stripes (3), with a transverse band (4) or variegated (5).
10. Lateral sepals widely spreading (0) or strongly incurved (1).
11. Petals (oblong-) obovate (0) or linear (1).
12. Petal ground colour pinkish to whitish (0) or yellowish to brownish (1).
13. Labellum glabrous to minutely papillose (0) or distinctly hairy (1).
14. Front edge of each labellum side lobe subequal to the hind edge (0) or much shorter than the hind edge (1) – cf. **Figure 1**.
15. Side lobes of labellum rounded to obtuse (0) or (sub)acute (1).
16. Mid-lobe of labellum (ob)ovate (0) or oblong to rounded (1).
17. Mid-lobe of labellum not distinctly thickened at base (0) or distinctly thickened at base (i.e. triangular in longitudinal section) (1).

18. Mid-lobe of labellum straight (0) or strongly recurved (1).
19. Abaxial angle between labellum mid-lobe and spur more than 90° (0) or up to 90° (1).
20. Spur cylindric to conical (0), pyriform to globular (1), hook-shaped (2) or spur absent (3).
21. Front wall of spur without (0) or with (1) a narrow longitudinal groove at its upper end.
22. A median callus at the base of the labellum mid-lobe absent (0) or present (1).
23. Spur not strongly recurved when dry (0) or spur strongly recurved when dry (1).
24. Back wall tongue in the spur absent (0) or present (1).
25. Back wall tongue placed near the spur apex (0) or more than 1/3 from the spur apex (1).
26. Back wall tongue truncate to obscurely emarginate (0) or distinctly bifid (1).
27. Margins of the back wall tongue only attached to the spur wall at its very base, not forming a distinct pouch (0); or margins of the back wall tongue adnate to the spur wall for a long distance, forming a pouch (1).
28. Pollinia two, porate (0) or pollinia four, entire, joined in two globular pairs (1).
29. Stipe spathulate (0) or oblong to linear (1).
30. Capsule up to 3 cm long (0) or more than 3 cm long (1).
31. Capsule distinctly stalked (0) or (sub)sessile (1).
32. Capsule glabrous to minutely papillose (0) or distinctly hairy (1).

Table 5. Character by taxon matrix of the analysis of intrageneric relationships. Characters as in **Table 1**. ? = unknown, - = inapplicable. Polymorphic taxa are coded with the following symbols; these are equate macros in PAUP: b = (0&1), d = (1&2), e = (0&2), f = (0&3).

	5	10	15	20	25	30	
Smitinandia	0b00d	b0000	000-0	10001	0000-	--100	10
Acampe	bbb0d	0b150	0111-	0bb00	0000-	--11b	10
Ventricularia	bb-00	10001	011-0	00002	0000-	--100	00
Haraella	01b00	01100	011?0	100-3	0000-	--01?	??
P. kunstleri	0b112	10bf0	10001	01b10	10011	10110	11
P. spicatum	0100d	100f1	010b0	00111	1b011	0b110	10
P. macphersonii	01001	00020	01011	01011	10011	01110	10

P. maculosum	1010d	0b020	01010	0b101	1b011	0111b	10
P. undulatum	01001	b0041	010b0	00111	bb011	10110	b0
P. bicolor	1010d	02120	01001	00111	11010	b1111	00
P. diffusum	b010d	0b010	01011	00111	1b011	bb11b	10
P. simalurense	10102	01b20	01001	00111	10110	01111	b0
P. fuscum	b0102	01020	0101b	00001	11011	b1110	10
P. marsupiale	b0102	0dbe0	01011	0b1b1	1b011	b1111	10
P. angustifolium	01001	00020	01011	00111	1b011	0b110	10
P. tokinense	01b0d	00130	10011	00110	11010	11110	10
P. floresanum	1010d	0b1?0	01001	00111	11010	b1111	10

Length of dorsal sepal (character 8). To avoid noise from expected allometry, this was the only floral size character to be included. In other words, the length of the dorsal sepal can be seen as a general measure of flower size.

Index of labellum side lobes (character 14). Nearly all *Pomatocalpa* species have labellum side lobes in which the hind edge produces a right angle to the front edge. The ratio of the lengths of the hind and front edges is informative. However, this character was coded as inapplicable for *P. undulatum* and *P. spicatum*, because the side lobes are broadly rounded in all specimens of the former and in some specimens of the latter.

Longitudinal section of labellum mid-lobe (character 17). This character was difficult to study from dried material. Therefore, it was only scored from fresh and spirit-preserved material.

A narrow longitudinal groove at upper front of the spur (character 21). In some specimens of *P. undulatum* the groove is distinct, while it is obscure in others. Consequently, the character was coded as polymorphic for this taxon.

B.3. *Phylogenetic analysis*

The phylogenetic analysis and the evaluation of internal support of clades were performed by the same methods as described for the intergeneric analysis based on morphological data (see above).

C. Total evidence

It was impossible to perform a congruency test on our data sets because of insufficient memory for simulation – probably due to the large proportion

of species for which DNA data were missing. However, since all the individual clades proved to have low bootstrap support, we found it justified to combine the data sets (in case of high bootstrap support for conflicting clades, data sets should not be combined; cf. de Queiroz 1993). Thus, a total evidence analysis of all data sets was performed to get the maximum information level (Kluge 1989). *Acampe ochracea*, *Smitinandia micrantha* and *Ventricularia tenuicaulis* were used as outgroups (since the DNA sequences were derived from individual species, these species, rather than entire genera, had to be chosen). For those species of *Pomatocalpa* from which molecular data were not available, the molecular characters were coded as missing.

Results

I. Delimitation and phylogenetic position of *Pomatocalpa* (morphological data)

Of the 18 characters scored, 14 were informative. The MP analysis yielded 3704 most parsimonious trees (length = 33; consistency index (CI) = 0.58; retention index (RI) = 0.73). The strict consensus tree is shown in **Figure 2**. The consensus tree suggests that *Pomatocalpa*, as traditionally circumscribed, is non-monophyletic. Most of the *Pomatocalpa* species, however, make up a monophyletic group. Bootstrap supports for branches in the strict consensus tree were less than 50% – except for the clade of *Micropera* and *Pomatocalpa bambusarum* (55%).

II. Interspecific relationships in *Pomatocalpa*

A. *Molecular data*

The *matK* alignment had a total of 1835 sites, out of which 110 were variable and 28 were phylogenetically informative. The MP analysis yielded 9 most parsimonious trees (length = 167; CI = 0.89; RI = 0.65). The strict consensus tree and the corresponding branch supports are shown in **Figure 3**. The resolution of the *matK* tree is very low. However, the clade made up from all the *Pomatocalpa* species in the analysis has strongly bootstrap support (91%), while the clade of *Haraella* and *Pomatocalpa* is moderately supported (79%).

The ITS alignment had a total of 668 sites, out of which 69 were variable and 24 were phylogenetically informative. The MP analysis yielded 6 most parsimonious tree (length = 116; CI = 0.85; RI = 0.64). The strict consensus tree and the corresponding branch supports are shown in **Figure**

4. The ITS tree is better resolved than the *matK* tree, but all clades are weakly bootstrap supported, and *Haraella retrocalla* is nested in *Pomatocalpa*.

B. *Morphology*

Out of the 32 characters scored, 28 were informative. The MP analysis yielded 108 most parsimonious trees (length = 63; CI = 0.61; RI = 0.69). The strict consensus tree with corresponding branch supports is shown in **Figure 5**. The following relationship has moderate bootstrap support, viz. the monophyly of *Pomatocalpa* (62%), the clade consisting of *P. simalurense*, *P. bicolor* and *P. floresanum* (68%), the sister group relationship of *P. kunstleri* and *P. tonkinense* (64%) and the sister group relationship of *P. spicatum* and *P. undulatum* (51%).

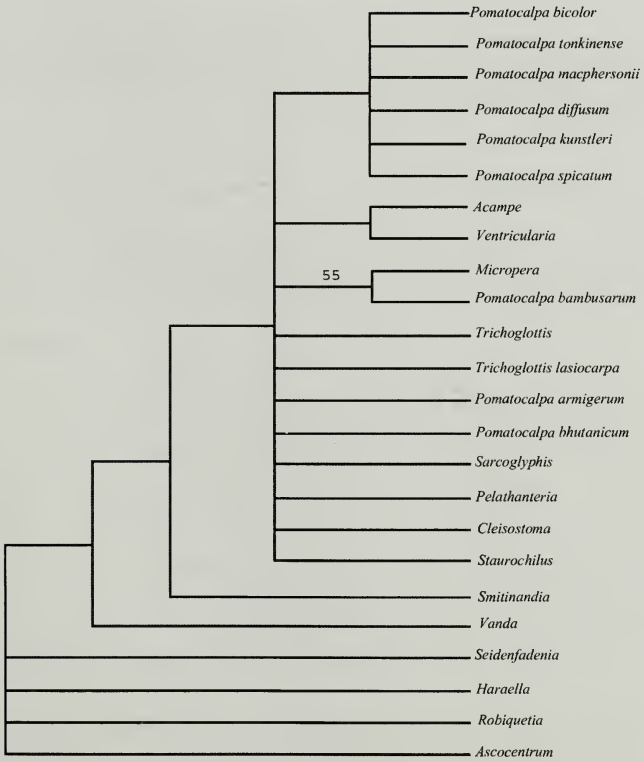

Figure 2. Strict concensus of the 3704 most parsimonious trees from the analysis of the phylogenetic position of *Pomatocalpa*, based on morphological data. Bootstrap support (if more than 50%) is indicated in percent above each branch.

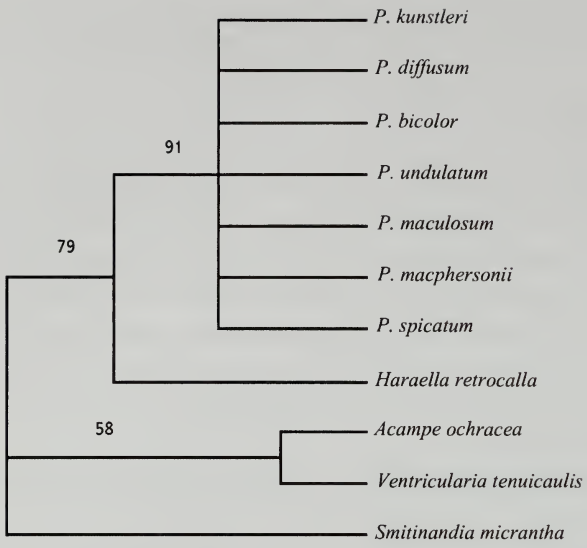

Figure 3. Strict consensus of the 9 most parsimonious trees from the analysis of the interspecific phylogenetic relationships in *Pomatocalpa*, based on *matK* data. Bootstrap support (if more than 50%) is indicated in percent above each branch.

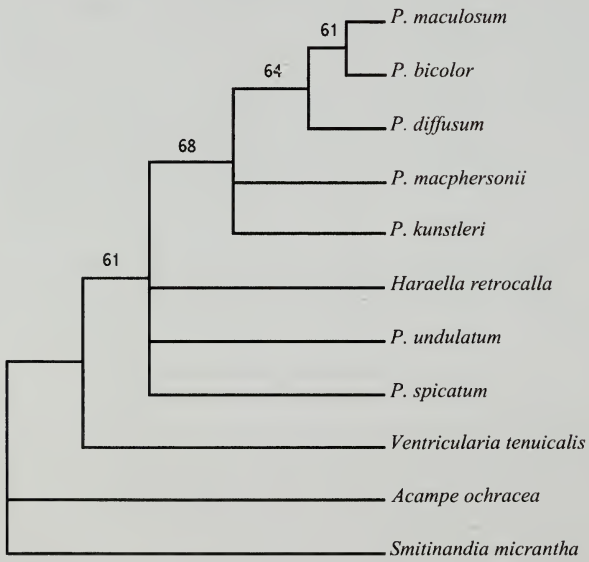

Figure 4. Strict consensus of the 6 most parsimonious trees from the analysis of the interspecific phylogenetic relationships in *Pomatocalpa*, based on ITS data. Bootstrap support (if more than 50%) is indicated in percent above each branch.

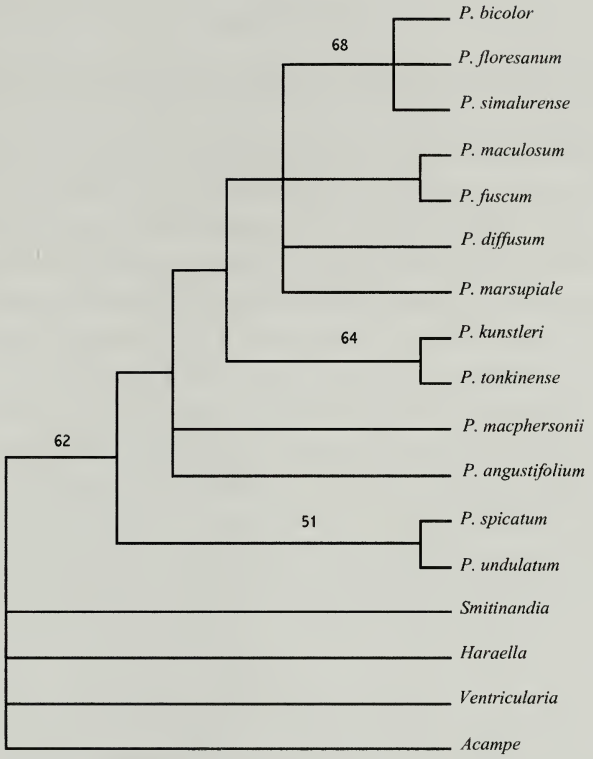

Figure 5. Strict consensus of the 108 most parsimonious trees from the analysis of the interspecific phylogenetic relationships in *Pomatocalpa*, based on morphological data. Bootstrap support (if more than 50%) is indicated in percent above each branch.

C. *Total evidence*

The data matrix of the combined molecular and morphological data sets has 2534 sites, of which 187 were variable and 82 were phylogenetically informative. The MP analysis yielded 45 most parsimonious trees (length = 365; CI = 0.81; RI = 0.60). The strict consensus tree with corresponding branch supports is shown in **Figure 6**. All analyzed *Pomatocalpa* species form a monophyletic group with 78% bootstrap support, as opposed to 62% in the morphological analysis. Also the clade consisting of this genus and its sister group, *Haraella retrocalla*, is fairly well supported (80%).

The interspecific relationships in *Pomatocalpa* are almost identical to the topology from the exclusively morphological analysis (except for the collapse of the clade containing *P. spicatum* and *P. undulatum*). The bootstrap support of the other clades within *Pomatocalpa* is slightly lower than in the morphological analysis.

Discussion

I. Phylogenetic position and achievement of monophyly of *Pomatocalpa*

The strict consensus tree from the phylogenetic analysis of representative *Pomatocalpa* species and a selection of closely related genera (**Figure 2**) indicates that *Pomatocalpa*, as traditionally circumscribed, is non-monophyletic. Thus, while most *Pomatocalpa* species make up a monophyletic group, three species are found outside this clade. *Pomatocalpa bambusarum* is sister to *Micropera*, while *P. armigerum* and *P. bhutanicum* form separate branches in the largely unresolved clade, which in addition to *Cleisostoma*, *Pelatantheria*, *Sarcoglyphis*, *Staurochilus* and *Trichoglottis*, also contains the monophyletic *Pomatocalpa* group and the *Micropera*/*Pomatocalpa bambusarum* clade. Unfortunately, we have been unable to obtain DNA data from these "misplaced" *Pomatocalpa* species. However, we tend to believe in the morphologically based phylogeny and find that *P. armigerum*, *P. bambusarum*, and *P. bhutanicum* should be removed to other genera to

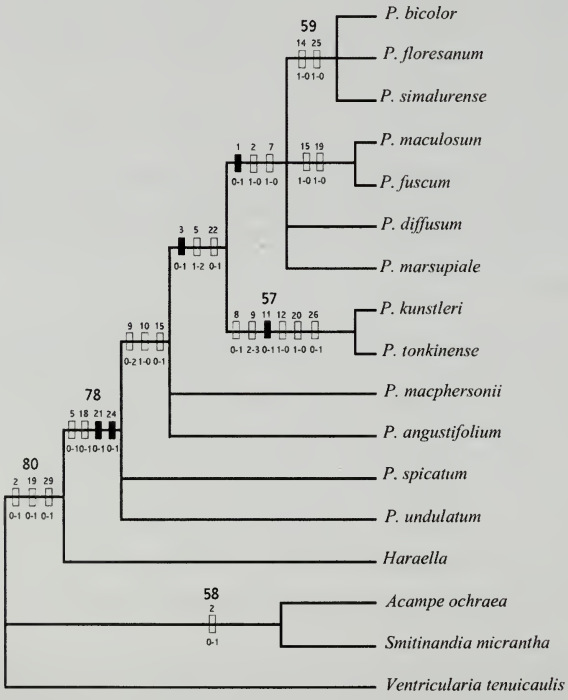

Figure 6. Strict consensus of the 45 most parsimonious trees from the analysis of the interspecific phylogenetic relationships in *Pomatocalpa*, based on total evidence (*matK*, ITS, morphology). The square boxes indicate the character numbers and character changes according to **Table 3** (black boxes: unique synapomorphies; white boxes: homoplacies). Bootstrap support (if more than 50%) is indicated in percent above each branch.

achieve monophyly of *Pomatocalpa* for the following reasons.

Pomatocalpa armigerum (King & Pantl.) T.Tang & F.Wang is probably better treated under its basionym, *Cleisostoma armigerum* King & Pantl., although its place in *Cleisostoma* is not unequivocal from the phylogenetic analysis in the present study (**Figure 2**). Just as *Pomatocalpa*, the very diverse genus *Cleisostoma* probably needs to be re-circumscribed. The back wall callus in the labellum spur of *C. armigerum* corresponds well with those of several species traditionally referred to *Cleisostoma*, e.g. *C. krabiense* (Seidenf.) Garay.

According to Pearce & Cribb (2002), Ormerod thinks that *Pomatocalpa bambusarum* (King & Pantl.) Garay might be better treated as *Cleisostoma bambusarum* (King & Pantl.) King & Pantl. According to the phylogenetic analysis in the present study (**Figure 2**), this species should rather be referred to *Micropera*. This, however, would call for a new combination. Therefore, we support the idea of tentatively referring the species to *Cleisostoma*, awaiting re-circumscription of the latter genus.

According to Pearce & Cribb (2002), Ormerod thinks that *Pomatocalpa bhutanicum* N.P.Balakr. might be better placed in *Cleisostoma*. We fully agree, admitting that its systematic position is not unequivocal from the phylogenetic analysis in the present study (**Figure 2**). The labellum shape and back wall callus in *P. bhutanicum* correspond well with those of several species traditionally referred to *Cleisostoma*. This calls for a new combination:

Cleisostoma bhutanicum (N.P.Balakr.) S.Watthana, *comb. nov.*–*Pomatocalpa bhutanicum* N.P. Balakr., J. Bombay Nat. Hist. Soc. 75: 162. 1978. – Type: Bhutan. Tashiyangtsi, 1870 m alt.; flowered in National Orchidarium, Shillong on 24 June 1965, *Balakrishnan* 41993 (holotype, CAL; isotype, ASSAM).

Likewise, judging from **Figure 2**, it seems appropriate that Ormerod (1997) transferred *Pomatocalpa lasiocarpa* to *Trichoglottis* – at least, this species clearly does not belong in *Pomatocalpa*.

When adjusted in this way, a slightly reduced, but monophyletic, *Pomatocalpa* can be readily recognized from a character state that provides a synapomorphy for the entire genus, i.e., the presence of a narrow longitudinal groove at the upper end of the front wall of the spur.

The phylogenetic relationships between *Pomatocalpa* and its neighbouring genera is very poorly resolved, and it is not unlikely that a

number of the genera used as Operational Taxonomic Unit (OTUs) in the present analysis would turn out to be polyphyletic themselves, if they were analyzed with species as OTUs. The very high number of equally most parsimonious trees is undoubtedly due to many cases of polymorphism as well as missing data (cf. Wilkinson, 1995). Phylogenetic analysis of the Aeridinae, based on *matK* and ITS sequences, are somewhat incongruent with macro-morphological data (Topik *et al.*, 2005). This is hardly surprising, since the majority of currently recognized genera under the Aeridinae are mainly, or exclusively, defined by floral traits (e.g. Garay, 1972, 1974; Senghas, 1986-1990; Seidenfaden, 1988). Floral characters are frequently found to show considerably high levels of homoplasy in the Orchidaceae (e.g. Pridgeon, et al. 1997; Bateman et al. 1997, 2003). Micromorphological features, such as the surface structure of velamen, pollen and seed, as well as sequence data from other genomic regions, could be interesting supplements in future analyses.

One of the neighbouring genera is of particular interest, i.e. the monotypic *Haraella*. As mentioned above, Topik and Yukawa (unpublished) conducted a precursory study which incorporated a large number of genera of the Aeridinae. They found *Pomatocalpa* (represented by five species) to be monophyletic in a phylogenetic analysis based on *matK* sequences. However, in the analysis based on ITS sequences, *Haraella* was nested in *Pomatocalpa*, rendering the latter paraphyletic. For this reason, *Haraella retrocalla* was included in the ingroup in each analysis in the present study. While both analyses based on morphology indicated only a distant relationship between *Pomatocalpa* and *Haraella* (**Figures 2, 5**), our analyses based on molecular data corroborated the preliminary findings (**Figures 3, 4**). According to our total evidence analysis, *Haraella* is not nested in *Pomatocalpa* (**Figure 6**).

We tend to assign most weight to the total evidence analysis, because combined data sets can increase the level of information (Kluge, 1989). Consequently, we accept that *Pomatocalpa* is monophyletic without *Haraella*. Acceptance on basis of total evidence seems to be reasonable due to relatively low bootstrap values in the separate analyses (cf. de Queiroz, 1993).

Indeed, only the consensus tree based on ITS sequence data suggests that *Pomatocalpa* and *Haraella* should be lumped to achieve monophyly of the former. The conflict between the strict consensus trees based on *matK* and ITS sequences, respectively, may be because *Haraella retrocalla* is of ancient hybrid origin. This hypothesis finds some support in a forthcoming

paper (Yukawa et al., in prep.) contributing further phylogenetic details on the Aeridinae. In that analysis, the Taiwanese endemic *Haraella retrocalla* is sister to a *Gastrochilus* species in the strict consensus tree based on *matK* data, while it is sister to the Taiwanese endemic *Pomatocalpa acuminatum* in the strict consensus tree based on ITS sequences. These different positions might well reflect ancient hybridization combined with maternal inheritance of cpDNA and biparental inheritance of ITS. However, the origin and phylogenetic affinities of *Haraella retrocalla* are in need of much closer scrutiny.

Even if someone finds the analysis based on ITS sequences more convincing than our other analyses, we think that transferring *Haraella* to *Pomatocalpa* would be controversial. Such an act would create a morphologically heterogenous genus that would not be recognizable as an entity outside the laboratory. Indeed, there are a number of good reasons to accept paraphyletic genera in such cases (e.g., Sosef, 1997; Brummitt, 2002, 2003; Grant, 2003). Having said this, we still think that there is fairly good support for considering *Pomatocalpa* a monophyletic genus.

II. Interspecific relationships in *Pomatocalpa*

While the interspecific relationships in *Pomatocalpa* are completely unresolved in our strict consensus tree based on *matK* sequences only (**Figure 3**), the consensus tree based exclusively on ITS data (**Figure 4**) suggests an infrageneric structure that is somewhat different from the one hypothesized by the consensus trees based on morphological data and total evidence, respectively. However, the clade containing *P. bicolor*, *P. diffusum* and *P. maculosum* is congruent with the morphological data and total evidence data.

The topology presented by the strict consensus tree based on total evidence (**Figure 6**) is almost identical to the consensus tree based on morphological data only (**Figure 5**). The only difference is that the clade containing *P. spicatum* and *P. undulatum* in the morphological tree (**Figure 5**) is found to be collapsed in the total evidence tree. The clade consisting of *P. kunstleri* and *P. tonkinense* has weak bootstrap support; "petals linear" being its only synapomorphy. The clade accommodating *P. bicolor*, *P. diffusum*, *P. floresanum*, *P. maculosum*, *P. marsupiale* and *P. simalurense* has bootstrap support less than 50%, but "stem more than 30 cm long" constitutes a synapomorphy (despite the fact that this character is variable in *P. diffusum*, *P. fuscum* and *P. marsupiale*). The clade consisting of *P. bicolor*, *P. floresanum* and *P. simalurense* also has weak bootstrap support,

with no unique synapomorphic character state. *P. maculosum* is sister to *P. fuscum* with no unique synapomorphy and the bootstrap support is more than 50%.

The strict consensus trees of the various analyses do not clearly reflect distribution patterns in *Pomatocalpa*. However, the clade consisting of *P. bicolor*, *P. floresanum* and *P. simalurense* seems confined to the Malesian region (occurring in Peninsular Malaysia, Indonesia and the Philippines) apart from an uncertain collection of *P. bicolor* from "Cochinchina".

In conclusion, neither our analyses of morphological data, nor *matK* or ITS sequence provided detailed resolution of the interspecific relationships in *Pomatocalpa* (**Figures 3, 4, 5**). Variation of *matK* and ITS at species level appeared to be very low, 6.0 % and 10.3 %, respectively. The lack of resolution is probably due to a high internal conflict among the sequences collected, as can be deduced from the relatively low RI values (0.65 and 0.64, respectively). To produce a more detailed phylogeny, it would be desirable to obtain more DNA data from additional species of *Pomatocalpa* as the high amount of missing molecular characters in the total evidence analyses is undoubtedly influencing the results of this study.

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